METHODS AND VACCINES FOR PROVIDING *IN OVO* PROTECTION AGAINST TURKEY RHINOTRACHEITIS

This application claims priority from copending provisional application serial number 60/252,162, filed on November 21, 2000, the entire disclosure of which is hereby incorporated by reference.

Field of the Invention

The invention is directed to useful methods for providing *in ovo* protection against turkey rhinotracheitis (TRT) and/or "Swollen Head Syndrome" (SHS) in avian hosts such as turkeys and chickens. More particularly, vaccines against TRT have proven to be both safe and efficacious upon appropriate *in ovo* administration to avian hosts as described herein.

Background of the Invention

TRT is an upper respiratory tract infection of turkeys that is caused by a pneumovirus. It is a highly contagious, acute disease that afflicts turkeys of all ages. The clinical symptoms of TRT infection include a marked, frequently frothy nasal discharge, rales, snicking, sneezing, and head shaking. Ocular discharge or swollen infraorbital sinuses may also be observed in infected turkeys.

Antibodies to TRT virus (TRTV) have been detected in some chicken flocks (both broilers and broilers/breeders) suffering from Swollen Head Syndrome (SHS). It is postulated that TRTV plays a role in the etiology of SHS and related respiratory distress.

Commercially-available vaccines for TRT are not administered *in ovo*. Rather, they are administered post-hatch in a variety of formats. Typically, such vaccines are administered by the labor-intensive methods of

spraying (*e.g.*, hand spray, knapsack spray, or automated spray equipment) or in drops (eye or nose).

As more fully explained below, the *in ovo* administration methods of using TRT vaccines modified for *in ovo* use provides distinctive advantages over the inconvenient and time-consuming post-hatch routes of administration presently available.

Summary of the Invention

The present invention utilizes commercially-available TRT vaccines adapted for the *in ovo* methods of administration of the present invention. Experimental results establish the safety and efficacy of the *in ovo* administration of these vaccines to turkeys and to chickens using appropriate dosing parameters.

The methods of the present invention can be utilized to protect an avian host against TRT, and/or TRT or SHS-related respiratory distress by *in ovo* administration of such vaccines.

It is thus an object of the present invention to provide a method of protection avian hosts from TRT and/or TRT or SHS-related respiratory distress using *in ovo* vaccination techniques which are easier and less expensive to apply to large populations of birds.

It is further an object of the present invention to provide such *in ovo* vaccination using vaccines in dosages which provide a suitable immunological response in hatched avian hosts without adversely affecting hatch rates.

It is a still further object of this invention to provide protection against SHS-related respiratory distress using TRT vaccines adapted for *in ovo* administration.

It is yet another object of the present invention to provide a method of vaccinating avian hosts against TRT and/or TRT or SHS-

respiratory distress which provides elevated titers to TRTV as compared to conventionally vaccinated birds, using lesser amounts of vaccine antigen, thus resulting in cost savings.

Detailed Description of the Invention

The present invention provides a method for immunizing *in ovo* avian hosts against TRTV, and thus providing protection against TRT and/or TRT and SHS respiratory distress. The vaccines utilized in the methods of the present invention can advantageously be prepared from commercially-available TRT vaccines. Especially suitable for use in the present invention is the commercially available Poulvac® TRT vaccine, available from Fort Dodge Animal Health, Fort Dodge, Iowa or Weesp, The Netherlands. The commercial formulation of Poulvac® TRT contains attenuated TRTV, strain K, with a titer of not less than $10^{3.2}$ $TCID_{50}$ per dose and is not approved or indicated for *in ovo* administration. Throughout this application, "$TCID_{50}$" refers to a 50% tissue culture infectious dose.

Typically, the vaccine is resuspended in a suitable vehicle so as to provide a $TCID_{50}$ in the range of about $10^{3.2}$ to about $10^{4.5}$, and administered in an amount of approximately 0.05 to 0.1 ml per egg, depending upon the avian species being immunized. Administration may be by hand, but is more typically and economically administered by using commercially available egg injection equipment such as that available from Embrex, Inc., North Carolina. The exact dosage to be administered will depend upon the avian species to which the vaccine is to be delivered, *e.g.*, smaller birds will require smaller doses. Administration of the vaccine typically occurs on or before day 24 of incubation (e.g., turkeys), but other *in ovo* vaccination times are within the scope of the invention, for example, on or before day 18 of incubation (e.g., chickens).

Avian hosts for which the vaccines and methods of the present invention are intended include chickens, ducks, turkeys, geese, bantams, quail and pigeons. Preferred avian species are the commercially important poultry birds such as chickens, ducks and turkeys.

It has surprisingly been found that not only is the *in ovo* method of vaccination safe and easier to administer, but that higher titers are found in avian hosts which have been immunized in this manner.

In addition, the vaccine and method of administration result in substantially no decrease in the percentage of eggs that hatch after *in ovo* vaccination, when compared to a substantially identical control (non-vaccinated) group. Preferably, this decrease is less than about 10%, and more preferably is less than about 5% relative to the percentage that hatch in the control group. Even more desirable is a decrease of less than about 1 - 2%. In some embodiments, the vaccine and method of the invention may actually increase the percentage of eggs that hatch, sometimes by as much as about 1-2% or even more. Thus, the vaccine is both safe and effective for administration to avian species such as chickens and turkeys.

The following examples describe in detail the methods and techniques illustrative of the present invention. It will be apparent to those skilled in the art that many modifications, both of materials and methods, may be practiced without departing from the purpose and intent of this disclosure.

EXAMPLES

Example 1- Safety study for *in ovo* turkey administration.

Fertile turkey eggs for hatching were obtained from parent turkey flocks which were known to be free of TRTV; and which had not been previously vaccinated against TRT. These eggs were randomly assigned to 2 different groups.

The first group of 76 fertile eggs was administered the vaccine *in*

ovo on day 24 of incubation. The first day of incubation is considered day 0. Eggs are laid approximately 2-7 days before incubation. Poulvac® TRT (batch TR015, expiration date 24 June 1997 containing a titer of 107.5 $TCID_{50}$) was used to prepare the *in ovo* vaccine. Three vials of the commercial product were each resuspended in 10 ml of sterile saline to give a resulting suspension having a titer of 105.5 $TCID_{50}$ of vaccine per 0.1 ml. These contents were well mixed and pooled. The mixed/pooled contents are redesignated as the "IOV" hereinafter in Example 1.

The *in ovo* administration used 0.1 ml of the IOV per egg containing a titer of 105.5 $TCID_{50}$, injected into the amniotic fluid of each of the 76 fertile eggs. Thereafter, these eggs were immediately placed into an incubator (without turning) and left to hatch in the isolation pen in which they were housed. These eggs/hatchlings are referred to as the vaccinated birds.

A second group of 66 fertile eggs were not vaccinated and were left to hatch under similar conditions in a second isolation pen. These eggs/hatchlings are referred to as the negative control birds.

For both the vaccinated birds and the negative control birds, hatching was recorded on days 27, 28, and 29 of incubation. Table 1 presents the experimentally-observed hatchability percentages. With respect to hatchability, the *in ovo* vaccination of the present invention produced excellent results with 93.4% of the vaccinated eggs hatching versus 92.4% of the negative control eggs hatching.

Table 1: Hatchability Percentages

Birds	Day 27	Day 28	Day 29	Total
Vaccinated	19.7%	73.7%	0%	93.4%
Negative Control	28.8%	63.6%	0%	92.4%

After hatching, 25 poults from each group (*i.e.*, the vaccinated birds and the negative control birds) were selected at random and placed on the floor on shavings in each of the respective isolation pens. The remaining

birds from each group were culled.

Within each group, each bird was examined daily for clinical signs for a period of 21 days. The presence of nasal exudate was assessed by squeezing the beak. The severity of clinical disease was scored according to Table 2.

Table 2: Clinical Scoring System

score	experimentally-observed symptoms
0	none
1	clear nasal exudate
2	turbid nasal exudate
3	swollen infraorbital sinuses or frothy eyes and 1 or 2

The total daily score of a group of birds was calculated by summarizing the individual scores of each bird on that day. Table 3 presents the experimentally-observed clinical signs using the relative scoring system of Table 2.

Table 3: Clinical Examination Results of Vaccinated Birds

	Age in days of the examined bird								
Bird #	3	4	5	6	7	8	9	10	11
76	0	0	0	0	0	0	0	0	0
77	0	0	1	1	0	0	0	0	0
78	0	0	1	0	2	0	0	0	0
79	0	0	0	2	0	0	0	0	0
80	0	0	0	0	0	0	0	0	0
81	0	0	0	0	0	0	0	0	0
82	0	0	0	0	0	0	0	0	0
83	0	0	0	0	0	0	0	0	0
84	0	0	0	0	0	0	0	1	0
85	0	0	0	0	0	0	0	0	0
86	0	0	0	0	0	0	0	0	0
87	0	0	0	2	1	2	0	0	0
88	0	0	0	0	0	0	0	0	0
89	0	0	0	0	0	0	0	0	0
90	0	2	2	2	2	0	0	0	0
91	0	0	0	1	2	2	2	0	0

92	0	0	0	0	0	0	0	0	0
93	0	0	0	0	0	0	0	0	0
94	0	0	0	0	0	0	0	0	0
95	0	0	0	0	0	0	0	0	0
96	0	0	0	0	0	0	0	0	0
97	0	0	0	1	0	0	0	0	0
98	0	0	0	0	0	0	0	0	0
99	0	0	0	0	0	0	0	0	0
100	0	0	0	0	0	0	2	0	0
Total	0	2	4	9	7	4	4	1	0
Mean Score/Bird	0	0.08	0.16	0.36	0.28	0.16	0.16	0.04	0
# Positive	0	1	3	6	4	2	2	1	0

The negative control birds were likewise examined for any clinical signs and, no abnormalities were observed, *i.e.*, all had a score of zero.

The results of the above observations establish the safety of the *in ovo* vaccination methods of the present invention. The highest Mean Score/Bird (*i.e.*, 0.36) for 6-day old birds provided an adequate margin of safety and indicated only slight/mild symptoms of TRT.

Serological analysis was also performed by collecting blood collected from 10 birds within the parent turkey flock 6½ weeks after the date that the eggs were received. Blood was also collected from the following 4 sets of birds: (a) at an age of 1 day, from 20 negative control birds; (b) at an age of 21 days, from 21 negative control birds; (c) at an age of 1 day, from 20 vaccinated birds; and (d) at an age of 21 days, from 20 vaccinated birds.

Serological analysis of the individual blood samples indicated titers of antibodies to TRTV. This analysis used an enzyme-linked immunosorbent assay (ELISA) technique which uses an A type antigen and expressed as ^{2}log titers. Experimentally-measured titers of ^{2}log titer ≥ 6.0 were taken to be positive. The geometrical mean (GM) and standard deviation (SD) for the experimentally-measured titers were also calculated. The titer analysis established the following results. The parent turkey flock was free of TRTV [see GM = 5.04 in comparison to the ≥ 6.0 positive cut-off value]. Of the 10

birds, one had an elevated titer of 7.7. Further, the GM titers for the 1-day old negative control birds and vaccinated birds were almost identical; *i.e.*, respectively, 4.05 and 4.06. However, two birds among the vaccinated birds had positive titers [see titer values of 7.1 and 8.1 - each ≥ 6.0]. By contrast, none of the negative control birds had titers ≥ 6.0. The SD for the negative control birds and the vaccinated birds were, respectively, 0.82 and 1.46.

The 21-day old blood sampling tests also showed clear differences in the experimentally-measured titers. For the negative control birds, the GM decreased to 3.53 and the SD decreased to 0.46. In direct contrast, for the vaccinated birds, the GM increased to 10.53 and the SD increased to 0.85. Accordingly, the vaccinated birds had greatly elevated titers to TRTV.

In the vaccinated birds, the serological response at an age of 21 days was very high. This response was even higher than that typically encountered using the same dose (via eye drop vaccination) for 1-day old birds. TRTV antibody titers having a GM = 10.5 are normally only seen following a challenge with a virulent strain of TRTV. For comparison, two trials using eye drop vaccinations with 105.5 $TCID_{50}$ Poulvac® TRT to susceptible turkey poults (at an age of 1 day) yielded mean antibody titers of, respectively, 8.3 and 8.7 for blood samples collected at an age of 21 days.

Table 4 presents the data supporting this serological testing of the parent turkey flock, the negative control birds, and the vaccinated birds.

Table 4: TRTV Titers

Parent Flock	Negative Control Birds		Vaccinated Birds	
6½ weeks	1-day age	21-day age	1-day age	21-day age
3.7	3.0	3.8	5.6	11.4
5.5	3.0	3.7	3.9	10.4
4.7	4.5	3.8	4.9	9.5
5.9	4.3	3.0	3.4	11.6
7.7	3.0	3.0	4.0	9.3

	5.6	4.8	3.4	3.0	9.9
	5.4	4.2	3.6	3.0	11.8
	3.7	4.7	3.6	3.0	11.1
	5.2	3.9	4.2	3.0	11.0
	3.0	3.0	4.5	7.1	9.7
		4.7	4.3	5.2	11.1
		5.0	3.2	3.9	9.1
		3.0	3.9	4.4	11.3
		4.8	3.0	3.0	10.6
		3.3	3.8	8.1	11.5
		5.1	3.5	3.3	11.2
		4.5	3.0	3.0	10.5
		4.1	3.0	3.0	10.0
		5.1	3.5	3.0	9.9
		3.0	3.3	3.5	9.6
			3.0		
GM	5.04	4.05	3.53	4.06	10.53
SD	1.35	0.82	0.46	1.46	0.85

Thus, *in ovo* administration (at day 24 of incubation) of an IOV of the present invention at 105.5 $TCID_{50}$ to fertile turkey eggs provides the necessary safety with respect to both hatchability and clinical signs, as well as an enhanced immune response over conventionally administered vaccines.

Example 2: Safety study for *in ovo* chicken administration.

Specific pathogen-free (hereinafter, "SPF) White Leghorn eggs were obtained from a commercial source (Broekman Instituut BV, Someren, The Netherlands). 120 SPF eggs were placed in an incubator, and after 18 days of incubation, the eggs were candled. This resulted in 5 non-fertilized eggs being rejected and 115 fertilized eggs being accepted. Of the 115 accepted eggs, 100 were randomly selected for *in ovo* vaccination. These 100 eggs were divided into three groups as follows:

Group 1 eggs/hatchlings were tagged for identification with an orange wing mark comprising a number. Group 1 consisted of 30 eggs

which, as described below, received an *in ovo* vaccine at a per egg calculated dose of a titer of 105.5 $TCID_{50}$.

Group 2 eggs/hatchlings were tagged for identification with a green wing mark comprising a number. Group 2 consisted of 40 eggs which, as described below, received an *in ovo* saline solution of equal volume to the vaccine injected to the Group 1 eggs.

Group 3 eggs/hatchlings were not tagged. Group 3 consisted of 30 eggs which did not receive any *in ovo* administration (either of the vaccine or of the saline solution).

The number on the wing marks was used only if the chick showed clinical signs of either TRT or SHS.

The chickens of both Groups 1 and 2 were housed in the same animal room in which they hatched. Appropriate conditions (*e.g.*, feed, drinking water, wood shavings as bedding materials, temperatures, relative humidities, etc.) were maintained.

The time schedule for this experiment was as follows: The first day of incubation of the eggs of Groups 1, 2, and 3 was termed day 0 of incubation. The date of *in ovo* administration to the eggs of Group 1 and Group 2 of, respectively, a vaccine and a saline solution was day 18 of incubation. The calculated hatching date corresponded to day 21 of incubation. The study was concluded on a post-hatch date equivalent to day 46 of incubation.

To prepare the vaccine for *in ovo* administration, a commercially-available vaccine, Poulvac® TRT containing 2000 doses per vial, available from Fort Dodge Animal Health in Fort Dodge, Iowa or Weesp, The Netherlands was used. On a per dose basis, this vaccine had a titer of $10^{4.2}$ $TCID_{50}$. Twelve (12) vials of this vaccine were resuspended in phosphate-buffered-saline (hereinafter, "PBS"), using 5 ml of PBS per vial of vaccine. The resulting contents were well mixed and pooled. The resuspended

material had a calculated titer of 105.5 $TCID_{50}$ and a total volume of 60 ml. This vaccine is hereinafter referred to as the "IOV".

On day 18 of incubation, the IOV vaccine was administered *in ovo* via injection using a commercially-available Inovoject® egg injection machine from Embrex, Inc., North Carolina to the eggs of Group I. The egg-injection administration of the IOV was conducted in accordance with standard procedures. In like manner, a commercially-available saline solution, CLEAR-FLEX® INFUSIEVLOESISTOF, from Bieffle Medital SpA, Italy was administered to the eggs of Group 2.

Table 5 shows the treatment of the eggs within Groups 1, 2, and 3.

Table 5: Treatment Of Eggs

Group #	# eggs/group	calculated dose per egg
1	30	$10^{5.5}$ $TCID_{50}$ vaccine
2	40	only saline solution
3	30	no treatment

The hatchability percentages of eggs from Groups 1, 2, and 3 were experimentally observed and calculated. In brief, the following exceptions were noted. Because of spina bifida skeletal abnormalities (which were not attributed to any adverse action of the *in ovo* vaccination), two chicks from Group 1 were removed from the study directly after they were hatched and before they were tagged with an identifying wing mark. These two chicks were excluded from the Group 1 calculated hatchability percentages. Also from Group 1, one chick was injured on the toes and removed from the study after it had been tagged. Post-mortem examination of this chick revealed no signs of either TRT or of any other disease or of any other disorders. This one chick was likewise excluded from the Group 1 calculated hatchability percentages. With respect to the eggs/hatchlings of Group 2, one chick died after being hatched but before being tagged with an

identifying wing mark. This chick was similarly excluded from the Group 2 calculated hatchability percentages.

As planned, the hatchlings of both Groups 1 and 2 were studied and examined for a 25-day observation period. During this period, none of the chicks showed clinical signs of either TRT or SHS. All 30 eggs of Group 3 hatched. On the day they hatched, all 30 chicks were decapitated for blood sampling and subsequent analysis as described later.

Table 6 presents the experimentally-observed hatchability and mortality results for each of Groups 1, 2, and 3. These results established that the *in ovo* vaccination of the present invention was safe with respect both hatchability and clinical signs of TRT and/or SHS.

Table 6: Hatchability/Mortality Results

	Group 1	Group 2	Group 3
	(orange wing mark)	(green wing mark)	(no wing mark)
	vaccine	saline	no treatment
Incubated	30	40	30
Vaccinated	30	40	0
Hatched	28	40	30
	Percentage of eggs:		
Vaccinated	100	100	0
Hatched	93.3	100	100

The body weights of the chicks from both Group 1 (vaccine) and Group 2 (saline) were obtained on day 25. Mean body weight of the Group 1 chicks was 209 grams with a standard deviation of 22.1. For the Group 2 chicks, the mean body weight was 217 grams with a standard deviation of 24.8. The body weights of Groups 1 and 2 did not differ significantly as statistically determined using a 2-sided Student's t test with $P = 0.18$). These results established that the *in ovo* vaccination of the present invention did not compromise the resulting day 25 body weight (which is commercially

important) in comparison to that obtained with an *in ovo* injection of a physiological saline solution.

To confirm the TRT-free status of the SPF eggs used in Groups 1, 2, and 3, on the day the Group 3 eggs hatched, the chicks were killed by decapitation and blood samples were collected and analyzed. ELISA testing did not detect any antibodies to TRTV which confirms the TRT-free status of the SPF eggs used in this study.

This study established that the *in ovo* vaccination to SPF chicken eggs was safe with respect to each of hatchability, mortality, clinical signs of TRT and/or SHS, and day 25 body weight.

Example 3: Efficacy study for *in ovo* SPF chicken vaccines.

The aim of this study was to ascertain whether *in ovo* vaccination of 18-day old-chicken embryos is efficacious in preventing TRT and/or SHS disease after virulent challenge at 3 or 6 weeks of age. As established below, *in ovo* vaccination of susceptible 18-day-old fertile SPF chicken eggs with $10^{4.2}$ $TCID_{50}$ is safe while a dose of $10^{3.2}$ $TCID_{50}$ is efficacious against clinical disease.

Fertile eggs for hatching were obtained from a flock of SPF White Leghorn parents purchased from Whickham Laboratories, United Kingdom.

A commercially-available TRT vaccine, Poulvac® TRT, available from Fort Dodge Animal Health in Fort Dodge, Iowa or Weesp, The Netherlands was obtained. The *in ovo* vaccines of the present invention are prepared from this Poulvac® TRT as follows. Three (3) vials of the commercial vaccine containing a titer of 107.5 $TCID_{50}$ were each resuspended in 4 ml of sterile water, and well mixed and pooled. Then 0.4 ml was removed and added to 19.6 ml of sterile PBS to give a final dilution equivalent to 200 ml per vial and a resulting suspension containing a titer of $10^{4.2}$ $TCID_{50}$ of vaccine per 0.1 ml. This vaccine was further diluted by

removing 2 ml and adding it to 18 ml of sterile PBS to give a resulting suspension of $10^{3.2}$ $TCID_{50}$ of vaccine per 0.1 ml.

The challenge virus was prepared as follows. TRTV from the UK strain BUT 8544, isolated by Dr. R.C. Jones at Liverpool University (U.K.), was passaged 23 times in trachea organ culture (hereinafter, "TOC"), once in poults, reisolated, and passaged once more in TOC. The titer of this challenge virus was $10^{4.5}$ $TCID_{50}$ per ml.

After 18 days of incubation, a first set of 70 fertile eggs (hereinafter, "Set 1") were inoculated *in ovo* with 0.1 ml of the reconstituted TRT vaccine containing $10^{3.2}$ $TCID_{50}$ as described above. A second set of 70 eggs (hereinafter, "Set 2") was likewise inoculated with 0.1 ml of the reconstituted TRT vaccine containing $10^{4.2}$ $TCID_{50}$ as described above. The eggs were immediately placed into an incubator (without turning) and left to hatch in the pen in which they were housed. The eggs of Sets 1 and 2 were housed separately in similar isolation pens. A third set of 70 fertile eggs did not receive any *in ovo* administrations (hereinafter, "Set 3"). Set 3 is hereinafter referred to as the negative control birds. These eggs were housed in a third isolation pen.

Hatching was recorded on days 20, 21, 22, and 23 post incubation (day zero is first day of incubation). After hatch, excess birds were culled at one-day-old to leave fifty birds per set. For Sets 1, 2, and 3, the experimentally-recorded hatchability percentages were, respectively, 91%, 94%, and 92%. This establishes that, *in ovo* vaccination with titers of $10^{3.2}$ $TCID_{50}$ and $10^{4.2}$ $TCID_{50}$ were safe with respect to hatchability.

At three weeks of age, ten birds from each vaccinated group (*i.e.*, Sets 1 and 2) and from the negative control birds (*i.e.*, Set 3) were wing-tagged and moved into a fourth isolation pen. Each bird was then administered with the previously described challenge virus via an eye drop containing a dose of $10^{3.5}$ $TCID_{50}$ (virulent) TRTV in 0.1 ml. At 6 weeks of

age, an additional 10 birds was likewise challenged with a virulent strain of TRTV. However, because of the increased age of these birds, the challenge dose was increased to 103.8 $TCID_{50}$ (virulent) TRTV in 0.2 ml on a per bird basis.

The challenged birds were experimentally monitored for 14 days, after which they were bled and killed. The observed signs were recorded using the Table 2 Clinical Scoring System as used in Example 1. The total daily score of a group of birds was calculated by summarizing the individual scores of each bird on that day. The cumulative score is the sum of the mean daily scores at days 3-8. The $\chi 2$ test was used to analyze the data. The total clinical signs seen in the 2 vaccinated groups (Sets 1 and 2) from days 3-8 was compared to those seen in the (Set 3) positive controls on the same days for both the 3-week and 6-week challenges. This monitoring established the following results.

With respect to the 3-week challenge with the virulent TRTV strain at a titer of $10^{3.5}$ $TCID_{50}$, 90% of the unvaccinated control birds of Set 3 showed clinical signs. In direct contrast, smaller percentages of the vaccinated birds of Sets 1 and 2 showed clinical signs. For Set 1 (vaccinated with a titer of $10^{3.2}$ $TCID_{50}$ per egg), only 50% of the birds showed clinical signs. For Set 2 (vaccinated with a titer of $10^{4.2}$ $TCID_{50}$ per egg), only 30% of the birds showed clinical signs. In Set 3, only one bird remained completely clear of clinical signs. In contrast, five birds from Set 1 and seven birds from Set 2 remained completely clear of clinical signs.

With respect to the 6-week challenge with the virulent TRTV strain at a titer of 103.8 $TCID_{50}$, 80% of the unvaccinated control birds of Set 3 showed clinical signs. In direct contrast, smaller percentages of the vaccinated birds of Sets 1 and 2 showed clinical signs. For Set 1 (vaccinated with a titer of $10^{3.2}$ $TCID_{50}$ per egg), only 20% of the birds showed clinical signs. For Set 2 (vaccinated with a titer of $10^{4.2}$ $TCID_{50}$ per egg), only 10% of

the birds showed clinical signs.

$\chi 2$ statistical analysis established, for the 6-week challenge, that the experimentally-observed clinical signs in both vaccinated groups of birds (Sets 1 and 2) were significantly less severe than those recorded in the unvaccinated negative control birds of Set 3 (see $P < 0.01$).

Tables 7, 8, and 9 present these results for, respectively, the 3-week challenge, the 6-week challenge, and the chi-squared statistical analysis as discussed above.

Table 7: Clinical Signs For 3-Week Challenge

	# of days post-challenge with virulent TRTV strain in eye drops at a dose of $10^{3.5}$ $TCID_{50}$ TRTV in 0.1 ml per bird							
	3	4	5	6	7	8	9	10
bird #	for Set 1 (vaccinated with $10^{3.2}$ $TCID_{50}$)							
104	0	2	2	2	0	0	0	0
106	0	0	2	0	0	0	0	0
107	0	0	0	0	0	0	0	0
111	0	2	0	0	0	0	0	0
113	0	0	0	0	0	0	0	0
117	0	0	0	0	0	0	0	0
118	0	1	2	2	0	0	0	0
124	0	0	0	0	0	0	0	0
127	0	0	0	0	0	0	0	0
139	0	0	1	2	0	0	0	0
Total Daily Score	0	5	7	6	0	0	0	0
Mean Daily Score	0	0.5	0.7	0.6	0	0	0	0
	Set 1 Cumulative Score per Bird = 1.8							
bird #	for Set 2 (vaccinated with $10^{4.2}$ $TCID_{50}$)							
3	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0
11	1	0	0	0	0	0	0	0
12	0	1	2	0	0	1	0	0
17	0	0	0	0	0	0	0	0
20	0	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0	0
29	0	0	0	0	0	0	0	0
36	2	1	2	2	0	0	0	0

37	0	0	0	0	0	0	0	0
Total Daily Score	3	2	4	2	0	1	0	0
Mean Daily Score	0.3	0.2	0.4	0.2	0	0.1	0	0
	Set 2 Cumulative Score per Bird = 1.2							
bird #	for Set 3 (not vaccinated)							
301	0	0	0	2	1	0	0	0
311	0	0	0	2	0	0	0	0
313	0	0	0	0	0	0	0	0
314	0	0	2	3	1	0	0	0
316	0	0	2	1	0	0	0	0
321	0	2	2	0	0	0	0	0
322	0	2	2	2	0	0	0	0
327	0	0	2	3	0	0	0	0
331	0	2	2	1	0	0	0	0
334	0	2	2	1	1	0	0	0
Total Daily Score	0	8	14	15	3	0	0	0
Mean Daily Score	0	0.8	1.4	1.5	0.3	0	0	0
	Set 3 Cumulative Score per Bird = 4.0							

Table 8: Clinical Signs For 6-Week Challenge

	# of days post-challenge with virulent TRTV strain in eye drops at a dose of $10^{3.8}$ $TCID_{50}$ TRTV in 0.2 ml per bird							
	3	4	5	6	7	8	9	10
bird #	for Set 1 (vaccinated with $10^{3.2}$ $TCID_{50}$)							
108	0	0	0	2	0	0	0	0
110	0	0	0	0	0	0	0	0
114	0	0	0	0	0	0	0	0
115	0	0	0	0	0	0	0	0
119	0	0	0	0	0	0	0	0
120	0	0	0	0	0	0	0	0
130	0	0	0	0	0	0	0	0
131	0	0	2	0	0	0	0	0
134	0	0	0	0	0	0	0	0
135	0	0	0	0	0	0	0	0
Total Daily Score	0	0	2	2	0	0	0	0

Mean Daily Score	0	0	0.2	0.2	0	0	0	0
	Set 1 Cumulative Score per Bird = 0.4							
bird #	for Set 2 (vaccinated with $10^{4.2}$ $TCID_{50}$)							
2	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0
13	0	0	0	0	0	0	0	0
18	0	0	2	2	0	0	0	0
25	0	0	0	0	0	0	0	0
27	0	0	0	0	0	0	0	0
32	0	0	0	0	0	0	0	0
38	0	0	0	0	0	0	0	0
39	0	0	0	0	0	0	0	0
Total Daily Score	0	0	2	2	0	0	0	0
Mean Daily Score	0	0	0.2	0.2	0	0	0	0
	Set 2 Cumulative Score per Bird = 0.4							
bird #	for Set 3 (not vaccinated)							
302	0	1	0	0	0	0	0	0
306	0	0	0	3	0	0	0	0
307	0	0	0	0	0	0	0	0
312	0	0	0	0	2	0	0	0
315	0	0	0	0	0	0	0	0
318	0	0	3	3	0	0	0	0
319	0	0	1	2	2	2	0	0
329	0	0	1	0	0	0	0	0
330	0	2	2	2	2	0	0	0
332	0	0	0	0	2	0	0	0
Total Daily Score	0	3	7	10	8	2	0	0
Mean Daily Score	0	0.3	0.7	1.0	0.8	0.2	0	0
	Set 3 Cumulative Score per Bird = 3.0							

Table 9: Chi-Squared Analysis

$\chi 2$ - Analysis of Clinical Scores: Comparison of 2 Vaccinated Groups (Sets 1 and 2) with 1 Negative Control Group (Set 3)			
Group	$\chi 2$	Deg. of Freedom	Probability
Set 1 (3-week challenge)	7.27	3	0.1>P>0.05

Set 2 (3-week challenge)	10.133	3	0.02>P>0.05
Set 1 (6-week challenge)	12.09	3	P<0.01
Set 2 (6-week challenge)	12.09	3	P<0.01

In addition to monitoring the birds of Sets 1, 2, and 3 for clinical signs of TRT, the birds were also subjected to serological analysis wherein antibodies to TRTV in individual blood samples were determined by ELISA techniques using an A type antigen and expressed as ^{2}log titers. Antibody titers of ^{2}log titer > 6.0 were taken to be positive. The results were statistically analyzed using a Student's t-test for unpaired data. Tables 10 and 11 present the serological results obtained with the challenged birds of Sets 1, 2, and 3 for, respectively, the 3-week challenge and the 6-week challenge. In Tables 10 and 11, "PC" refers to post-challenge; "GM" refers to geometric mean; and "SD" refers to standard deviation. In brief, all the groups (*i.e.*, Sets 1, 2, and 3) showed a significant rise in antibody titers at 7 post-challenge days and a additional rise in antibody titers at 14 days PC.

Table 10: TRTV Antibody Titers (3-Week Challenge)

	^{2}log antibody titers after challenge with virulent TRTV in eye drops at a dose of $10^{3.5}$ $TCID_{50}$ in 0.1 ml per bird		
	prior to challenge	7 days PC	14 days PC
bird #	for Set 1 (vaccinated with $10^{3.2}$ $TCID_{50}$)		
104	4.4		9.9
106	3.6	9	9.9
107	6.2	7	9.5
111	6.6	10.4	11.2
113	3.4		9.4
117	3.5		8.8
118	3.6	6.4	9.5
124	3.2	6.8	9.7
127	10.1		10.9
139	3.4		10.2
GM	4.8	7.92	9.9

SD	2.22	1.71	0.72
bird #	for Set 2 (vaccinated with $10^{4.2}$ $TCID_{50}$)		
3	4.4	9	9.9
6	9.7		10.6
11	4.6	10.3	10.9
12	4.3	7.5	10
17	8.4	10.6	11.1
20	9.1		
21	4.5		10
29	4.4		10
36	3.6	9.6	10
37	4.8		10.1
GM	5.78	9.4	10.29
SD	2.31	1.23	0.45
bird #	for Set 3 (not vaccinated)		
301	3.3		9.2
311	3.4	9.1	9.9
313	3	9	9.7
314	3.7	9.9	10.3
316	3.7		10.2
321	4.2	9.5	9.8
322	3.9	7.7	10
327	3.4		10
331	3.5		10.5
334	3.5		10.3
GM	3.56	9.04	9.99
SD	0.28	0.83	0.37

Table 11: TRTV Antibody Titers (6-Week Challenge)

	[2]log antibody titers after challenge with virulent TRTV in eye drops at a dose of $10^{3.8}$ $TCID_{50}$ in 0.2 ml per bird		
	prior to challenge	7 days PC	14 days PC
bird #	for Set 1 (vaccinated with $10^{3.2}$ $TCID_{50}$)		
108	4		10.2
110	5.9		10.1
114	4.7		9.8
115	4	6	9.9
119	4.1	6.5	10.7
120	4		10.4
130	3.9		9.8

131	3.5	7	9.9
134	3.9	6.4	9.6
135	4	5.5	10
GM	4.2	6.28	10.04
SD	0.67	0.56	0.32
bird #	for Set 2 (vaccinated with $10^{4.2}$ $TCID_{50}$)		
2	5.8		10
4	7.1		9.3
8	6.4	10.3	10.4
13	5.2		10.2
18	8.6	10.2	11.2
25	10.4	10.7	10.9
27	10.2	10.5	11.4
32	10.3		
38	10.8	10.9	11.1
39	6.8		9.5
GM	8.16	10.52	10.47
SD	2.14	0.29	0.72
bird #	for Set 3 (not vaccinated)		
302	3.8	7.1	10.4
306	4.4	8.1	10.1
307	4.1		10.1
312	3.8	6.3	9.9
315	4.1	6	9.9
318	3.9		10
319	4.2		9.9
329	3.8		9.9
330	4.3	7.7	9.7
332	3.4		9.9
GM	3.98	7.04	9.98
SD	0.30	0.89	0.19

A similar serological analysis was also performed with respect to all three groups (*i.e.*, Sets 1, 2, and 3) which were not challenged with a virulent strain of TRTV. Mean antibody titers from the control birds (Set 3) and from the 2 groups of vaccinated birds (Sets 1 and 2) from one-day old to 8½-weeks old were determined. As expected, the antibody titers for the control birds remained low throughout this study. The titers of the control birds were statistically compared to those of the vaccinated birds of the same

age. Table 12 presents the post *in ovo* determined mean antibody titers. Individual results were available (data not shown) to support the experimental results presented in Table 12. In Table 12, "n" refers to the number of birds and "SD" refers to the Standard Deviation.

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Table 12: Post In Ovo Mean Antibody Titers

	n	SD	Mean Antibody Titer
Age	for Set 1 (vaccinated with $10^{3.2}$ $TCID_{50}$)		
1-Day Old	10	0.87	4.95
1 Week	5	0.51	4.06
2 Weeks	5	1.54	3.88
3 Weeks	20	1.71	4.06
4 Weeks	5	0.22	4.28
5 Weeks	10	0.67	4.55
6 Weeks	10	0.67	4.2
8½ Weeks	10	2.04	5.83
Age	for Set 2 (vaccinated with $10^{4.2}$ $TCID_{50}$)		
1-Day Old	11	0.56	4.3
1 Week	5	0.45	4.26
2 Weeks	5	2.82	4.76
3 Weeks	20	2.42	5.98
4 Weeks	5	2.98	7.16
5 Weeks	10	2.38	7.03
6 Weeks	10	2.12	8.14
8½ Weeks	9	2.56	8.13
Age	for Set 3 (not vaccinated)		
1-Day Old	10	0.73	3.88
1 Week	5	0.96	4.72
2 Weeks	5	0.26	3.8
3 Weeks	18	0.27	3.49
4 Weeks	5	0.17	4.4
5 Weeks	11	0.55	3.61
6 Weeks	11	0.35	3.92
8½ Weeks	9	0.86	3.98

An analysis of the results of Example 3, including the experimentally-obtained measurements, results, and corresponding statistical

analysis, indicates that the lower dose of vaccine ($10^{3.2}$ $TCID_{50}$ TRTV delivered *in ovo* to susceptible 18-day-old fertile SPF chicken eggs) was efficacious in that it conferred significant protection against challenge with a virulent strain of TRTV at 6 weeks of age. At 3 weeks of age, protection was also observed; however, in light of the number of birds, the experimentally-observed differences in the protection afforded fell just beneath the level of significance. At the increased vaccination dose of $10^{4.2}$ $TCID_{50}$ TRTV per egg (an increase on the order of approximately ^{1}log10), for both the 3-week and the 6-week challenges, significant protection against exposure to a virulent strain of TRTV was observed.

In ovo administration of the vaccine derived from the Poulvac® TRT was clearly associated with the production of TRTV-specific antibodies in the vaccinated/inoculated birds. This association appeared to be dose-dependent. The higher dose of the *in ovo* vaccine caused an increase in mean antibody titer at three weeks of age to levels significantly higher than those of the negative control birds and to levels closely below the cutoff value of 6.0. The circulating antibody response to the *in ovo* vaccination with the higher dose of $10^{4.2}$ $TCID_{50}$ per egg appeared to increase with the passage of time; a maximum was obtained at six weeks of age; and, thereafter, the titers remained steady until 8½ weeks of age.

In contrast to the above discussed results for the high vaccination dose, the birds given the lower dose of $10^{3.2}$ $TCID_{50}$ per egg appeared to experience a delayed antibody response to vaccination. Titers only started increasing at 8½ weeks of age - when they reached levels significantly higher than those of the control birds. At this time (*i.e.*, 8½ weeks) the titers for the low vaccine birds (Set 1) were at similar levels to those of the high vaccine birds (Set 2) at an age of 3 weeks. It appeared likely that, with the passage of yet additional time beyond 8½ weeks, the antibody titers would have increased to levels above 6.0.

A relationship appeared between antibody titers and reduction in clinical signs of TRT. In each grouping of birds where the antibody titers were significantly higher than those of the control birds but nonetheless below the positive cutoff value of 6.0; - the observed reduction in clinical signs of TRT was statistically significant.

In ovo vaccination to susceptible day-18-old fertile SPF chicken eggs with doses in the approximate range of from at least $10^{3.2}$ $TCID_{50}$ per egg to at least $10^{4.2}$ $TCID_{50}$ per egg, and, in particular, with doses of approximately $10^{4.2}$ $TCID_{50}$ per egg appeared both safe and efficacious against clinical disease normally expected from challenge with a virulent strain of TRTV.

Example 4: Efficacy study for *in ovo* commercial chicken vaccines.

The aim of this study was to ascertain whether *in ovo* vaccination of 18-day-old incubated fertile chicken eggs from a parent flock of commercial broilers is efficacious in preventing TRT and/or SHS disease after virulent challenge at 4 or 6 weeks of age. As established below, *in ovo* vaccination of susceptible 18-day-old fertile eggs from TRTV-antibody-positive parents with $10^{3.2}$ $TCID_{50}$ per egg with a vaccine derived from Poulvac® TRT is efficacious against clinical rhinotracheitis disease.

Fertile eggs for hatching were obtained from a flock of 37-week-old commercial broiler parents which had been previously vaccinated with live TRT vaccine at 10 weeks of age and with killed TRT vaccine at 18 weeks of age. These eggs were obtained from the Mossbank breeder flock, Marshall Agriculture, Whitburn, Scotland.

A commercially-available TRT vaccine, Poulvac® TRT, available from Fort Dodge Animal Health, Fort Dodge, Iowa or Weesp, The Netherlands was used to prepare the *in ovo* vaccines of the present invention. Three vials of this commercial vaccine containing a titer of 107.5

$TCID_{50}$ were each resuspended in 5 ml of sterile water, and the contents well mixed and pooled. The vaccine was further diluted in sterile phosphate-buffered saline (PBS) to give a resulting suspension of $10^{3.2}$ $TCID_{50}$ of vaccine per 0.1 ml.

The challenge virus was prepared as follows. TRTV from the UK strain BUT 8544 (see page 12) was passaged 23 times in trachea organ culture (hereinafter, "TOC"), once in poults, reisolated, and passaged once more in TOC. The titer of this challenge virus was $10^{4.5}$ $TCID_{50}$ per ml.

After 18 days of incubation, 57 fertile eggs were inoculated *in ovo* with 0.1 ml of the reconstituted TRT vaccine containing $10^{3.2}$ $TCID_{50}$ as described above. The eggs were immediately placed into an incubator (without turning) and left to hatch in the isolation pen in which they were housed. After hatching, 40 birds were removed from the incubator and placed on the floor on shavings.

A number (110) of fertile eggs were not vaccinated/inoculated and were left to hatch separately. A day after hatching, forty birds were housed in a second isolation pen as the negative control birds. Challenged control birds were called positive control birds.

Hatching was recorded on days 20, 21, 22, and 23 (inoculation day - zero). After hatch, excess birds were humanely killed or used for the collection of blood. The hatchability percentages for the non-vaccinated eggs and the vaccinated eggs were, respectively, 89% and 91% which establishes that, with respect to hatchability, vaccination with a titer of $10^{3.2}$ $TCID_{50}$ was safe.

At 4 weeks of age, ten birds from the vaccinated group and ten birds from the negative controls were wing-tagged and moved into a third isolation pen. Each bird was then administered with the previously described challenge virus via an eye drop containing a dose of $10^{3.5}$ $TCID_{50}$ (virulent) TRTV in 0.1 ml. At 6 weeks of age, an additional 14 birds was likewise

challenged with a virulent strain of TRTV. However, because of the increased age of these birds, the challenge dose was increased to 103.8 $TCID_{50}$ (virulent) TRTV in 0.2 ml, on a per bird basis.

The challenged birds were experimentally monitored for 14 days, after which they were bled and killed. The observed signs were recorded using Table 13 Revised Clinical Scoring System. The Table 13 system was similar to but not identical with the Table 2 system previously described in Example 1 above.

Table 13: Revised Clinical Scoring System

Score	experimentally-observed symptoms
0	no signs
1	clear nasal exudate
1	frothy eyes but no nasal exudate (F)
2	turbid nasal exudate
3	swollen infraorbital sinuses or frothy eyes and 1 or 2

The total daily score of a group of birds was calculated by summarizing the individual scores of each bird on that day. The cumulative score is the sum of the mean daily scores. The $\chi 2$ test was used to analyze the data. The total clinical signs seen in the Set 1 vaccinated group was compared to those seen in the Set 2 positive controls on the same days for both the 4-week and 6-week challenges. This monitoring established the following results.

With respect to the 4-week challenge with the virulent TRTV strain at a titer of $10^{3.5}$ $TCID_{50}$, clinical signs were only observed on days 6 and 7, - the maximum signs were seen on day 6 in both groups (*i.e.*, Sets 1 and 2). In the vaccinated group (Set 1), only 30% of the birds exhibited clinical signs after challenge and the cumulative mean score per bird was 0.6. In Set 1, seven out of ten birds remained completely clear of clinical signs. The total clinical signs seen in the vaccinated birds were statistically compared to

those of the positive control birds. Although the vaccinated birds exhibited less severe clinical signs than those of the positive control birds, these results were not statistically significant ($0.2<P<0.3$).

With respect to the 6-week challenge with the virulent TRTV strain at a titer of 103.8 $TCID_{50}$, clinical signs were observed for longer periods of time in the positive control birds than after the 4-week challenge with the lower dose. However, in the vaccinated birds, clinical signs were observed for only one day. Of the positive control birds, 57% showed clinical signs and the cumulative score per bird was 1.35. Of the 14 birds in this group, 6 remained completely clear of clinical signs. One bird showed severe clinical signs on days 5 and 6 of the observation period and subsequently died nine days after challenge. Post-mortem examination of this bird revealed a dilated right ventricle, congested cardiac veins, lung congestion, excess mucous in the trachea, and a fibrinous exudate on the liver. The probable cause of death was right heart failure and possible hepatitis. By contrast, only 14% of the vaccinated group of birds exhibited clinical signs after challenge and the cumulative score per bird was 0.14. Of the 14 birds in this group, 12 remained completely clear of clinical signs. The total clinical signs in the vaccinated group (Set 1) was statistically compared using a $\chi 2$ analysis to those seen in the positive control birds (Set 2). This analysis established that the clinical signs seen in the vaccinated group were significantly less severe than those seen in the positive control birds ($0.02<P<0.05$).

Tables 14, 15, and 16 present these results for, respectively, the 4-week challenge, the 6-week challenge, and the $\chi 2$ statistical analysis as discussed above.

Table 14: Clinical Signs For 4-Week Challenge

	# of days post-challenge with virulent TRTV strain in eye drops at a dose of $10^{3.5}$ TRTV $TCID_{50}$ in 0.1 ml per bird							
	3	4	5	6	7	8	9	10
bird #	for Set 1 (vaccinated with $10^{3.2}TCID_{50}$)							
41	0	0	0	0	F	0	0	0
42	0	0	0	0	0	0	0	0
43	0	0	0	2	0	0	0	0
44	0	0	0	0	0	0	0	0
45	0	0	0	0	0	0	0	0
46	0	0	0	0	0	0	0	0
47	0	0	0	0	0	0	0	0
48	0	0	0	2	F	0	0	0
49	0	0	0	0	0	0	0	0
50	0	0	0	0	0	0	0	0
Total Daily Score	0	0	0	4	2	0	0	0
Mean Daily Score	0	0	0	0.4	0.2	0	0	0
	Set 1 Cumulative Score per Bird = 0.6							
bird #	for Set 2 (nonvaccinated)							
391	0	0	0	3	0	0	0	0
392	0	0	0	0	0	0	0	0
393	0	0	0	0	0	0	0	0
394	0	0	0	2	0	0	0	0
395	0	0	0	3	F	0	0	0
396	0	0	0	0	F	0	0	0
397	0	0	0	0	0	0	0	0
398	0	0	0	1	F	0	0	0
399	0	0	0	1	0	0	0	0
400	0	0	0	0	0	0	0	0
Total Daily Score	0	0	0	10	3	0	0	0
Mean Daily Score	0	0	0	1	0.3	0	0	0
	Set 2 Cumulative Score per Bird = 1.3							

Table 15: Clinical Signs For 6-Week Challenge

	# of days post-challenge with virulent TRTV strain in eye drops at a dose of 103.8 TRTV $TCID_{50}$ in 0.2 ml per bird							
	3	4	5	6	7	8	9	10
bird #	for Set 1 (vaccinated with $10^{3.2}TCID_{50}$)							
1	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0
52	0	0	0	0	0	0	0	0
53	0	0	0	0	0	0	0	0
54	0	0	0	0	0	0	0	0
55	0	0	0	0	0	0	0	0
56	0	0	0	0	0	0	0	0
57	0	0	0	0	0	0	0	0
59	0	0	0	F	0	0	0	0
60	0	0	0	0	0	0	0	0
62	0	0	0	F	0	0	0	0
65	0	0	0	0	0	0	0	0
69	0	0	0	0	0	0	0	0
Total Daily Score	0	0	0	2	0	0	0	0
Mean Daily Score	0	0	0	0.14	0	0	0	0
	Set 1 Cumulative Score per Bird = 0.14							
bird #	for Set 2 (nonvaccinated)							
381	0	0	0	0	0	0	0	0
383	0	0	0	0	0	0	0	0
387	0	0	0	0	0	0	0	0
388	0	F	0	0	0	0	0	0
389	0	0	0	0	0	1	0	0
390	0	0	F	0	0	0	0	0
463	0	0	2	F	0	1	0	0
464	0	0	0	0	0	0	0	0
465	0	0	0	2	0	0	0	0
469	0	0	0	0	0	1	0	0
474	0	0	0	0	0	0	0	0
476	0	0	3	3	0	0	dead	0
477	0	0	0	0	0	0	0	0
478	0	0	2	F	0	0	0	0
Total Daily	0	1	8	7	0	3	0	0

Score								
Mean Daily Score	0	0.07	0.57	0.5	0	0.21	0	0
	Set 2 Cumulative Score per Bird = 1.35							

Table 16: Chi-Squared Analysis Of Clinical Score Data

$\chi 2$ – Analysis of Clinical Scores: Comparison of 1 Vaccinated Group (Set 1) with 1 Positive Control Group (Set 2)			
Group	$\chi 2$	Deg. of Freedom	Probability
Set 1 (4-week challenge)	3.95	3	0.2<P<0.3
Set 1 (6-week challenge)	8.43	3	0.02<P<0.05

In addition to monitoring the birds of Sets 1 and 2 for clinical signs of TRT, the birds were also subjected to serological analysis wherein antibodies to TRTV in individual blood samples were determined by ELISA techniques developed at Leahurst, Liverpool, (U.K.) using an A type antigen and expressed as ^{2}log titers. Antibody titers of ^{2}log titer > 6.0 were taken to be positive. The results were statistically analyzed using a Student's t-test for unpaired data. Tables 17 and 18 present the serological results obtained with the challenged birds of Sets 1 and 2 for, respectively, the 4-week challenge and the 6-week challenge. In Tables 17 and 18, "PC" refers to post-challenge.

Table 17: TRTV Antibody Titers (4-Week Challenge)

	^{2}log antibody titers after challenge with virulent TRTV in eye drops at a dose of $10^{3.5}$ $TCID_{50}$ in 0.1 ml per bird	
	prior to challenge	14 days PC
bird #	for Set 1 (vaccinated with $10^{3.2}TCID_{50}$)	
41	3.0	9.4
42	3.0	9.2
43	3.0	10.1

44	3.0	9.5
45	6.9	10.8
46	5.4	11.2
47	3.0	10.2
48	3.0	10.6
49	8.2	9.9
50	9.2	11.0
Mean	4.77	10.19
Standard Deviation	2.48	0.70
bird #	for Set 2 (nonvaccinated)	
391	3.3	9.5
392	3.5	10.3
393	3.0	9.7
394	3.1	9.1
395	3.6	8.9
396	3.6	9.7
397	3.1	9.8
398	3.4	9.3
399	3.0	9.7
400	3.4	9.9
Mean	3.3	9.6
Standard Deviation	0.24	0.41

Table 18: TRTV Antibody Titers (6-Week Challenge)

	^{2}log antibody titers after challenge with virulent TRTV in eye drops at a dose of $10^{3.8}$ $TCID_{50}$ in 0.2 ml per bird	
	prior to challenge	14 days PC
bird #	for Set 1 (vaccinated with $10^{3.2}TCID_{50}$)	
1	6.3	11.6
2	4.3	10.4
3	4.2	10.1
52	3.6	10.6
53	4.1	10.6
54	3.7	10.7
55	4.8	9.7
56	3.6	9.9
57	8.1	10.1
59	3.4	10.2
60	3.6	9.8

62		9.3
65	5.3	10.0
69	4.9	10.1
Mean	4.61	10.22
Standard Deviation	1.34	0.55
bird #	for Set 2 (nonvaccinated)	
381	4.2	9.8
383	5.2	9.2
387	3.0	9.9
388	3.4	9.6
389	3.8	9.0
390	5.4	8.8
463	4.0	10.0
464	3.0	9.7
465	3.7	8.7
469	3.0	9.6
474	3.9	9.9
476	4.7	
477	4.8	9.9
478	5.0	9.6
Mean	4.08	9.52
Standard Deviation	0.83	0.44

A similar serological analysis was also performed with respect to those birds which were not challenged with a virulent strain of TRTV, using ten serum samples taken from birds from each of 5 different air spaces of the Mossbank parent breeder flocks (Marshalls Agriculture) at 25 and 41 weeks of age. Table 19 presents the mean antibody titers of these birds from the parent breeder flock.

Table 19: Mean Antibody Titers Of Parent Breeder Flock

	25 weeks old (n = 10)		41 weeks old (n = 10)	
house	Mean	SD	Mean	SD
4A	7.60	2.57	7.39	1.72
5A	7.51	0.81	8.27	0.87

3B	7.44	2.55	7.29	1.50
4B	8.16	0.67	6.64	1.46
5B	7.34	1.81	7.17	0.98

For both the unchallenged vaccinated and the unchallenged negative control birds, blood was collected at ages of 1-day-old and 4, 6, and 8 weeks of age. Table 20 presents this mean antibody titers for the unchallenged birds.

Table 20: Mean Antibody Titers For Unchallenged Birds

	nonvaccinated negative control birds			vaccinated birds		
age	mean	SD	n	mean	SD	n
1-day	7.23	1.32	10	6.46	0.99	10
4 weeks	3.52	0.54	38	4.38	1.86	39
6 weeks	3.93	0.72	28	4.72	1.62	27
8 weeks	3.54	0.75	14	5.09	1.68	14

For both the challenged vaccinated and the challenged positive control birds, blood was collected 14 days post-challenge for each of the 4-week and the 6-week challenged/groups. Table 21 presents this mean antibody titers for the challenged birds in these 2 groups.

Table 21: Titers For Challenged Birds At 14 Days PC

	4-week challenge			6-week challenge		
group	mean	SD (P)	n	mean	SD (P)	n
positive control birds	9.6	0.41 (-)	10	9.52	0.44 (-)	13
vaccinated birds	10.19	0.70 (0.031)	10	10.22	0.55 (0.001)	14

Individual results were available to support the data presented above in Tables 14-21. Based on the above serological analysis, the following conclusions were made. For both the vaccinated birds and the negative control birds, at an age of 1 day, these birds possessed maternal antibodies (MA) to TRTV. By 4 weeks of age, antibody levels in the negative control birds had dropped to the negative range and remained low throughout the remainder of the experiment. Following *in ovo* administration with a titer of $10^{3.2}$ $TCID_{50}$ of a vaccine derived from Poulvac® TRT, mean antibody titers remained in the negative range (*i.e.*, below the positive cutoff value of 6.0) but increased with age. At 4, 6, and 8 weeks of age, the vaccinated birds possessed mean antibody titer levels which were statistically significantly higher than those of the negative control birds. From 4 weeks of age, 20% to 22% of the vaccinated birds had positive titers. With respect to the challenged birds, all birds showed seroconversion at 14 days post-challenge. For both the 4-week and the 6-week challenge studies, the mean titers of the vaccinated group were higher than those of the positive control birds and this difference was statistically significant ($P < 0.05$).

An analysis of the entirety of Example 4, including the experimentally-obtained measurements, results, and corresponding statistical analysis indicates that *in ovo* vaccination of maternal-antibody-positive (MA+) commercial broiler eggs at 18 days incubation with a vaccine derived from Poulvac® TRT at a dosage titer of $10^{3.2}$ $TCID_{50}$ per egg did not adversely effect hatchability and provided a reliable, efficient, and efficacious method of vaccine administration. This vaccination also conferred significant protection against challenge with a virulent strain of TRTV at 6 weeks of age - when the clinical signs seen were significantly reduced. There was a degree of protection at 4-weeks of age - however, even in the nonvaccinated positive control birds the clinical signs seen at this time were not very severe. Accordingly, although present, the protection afforded against a challenge at

4-weeks was not statistically significant.

The data presented in this Example 4, in combination with that of Examples 1-3 above, established that chickens are far less susceptible to TRT vaccines. Positive mean antibody titers (above the positive cutoff value of 6.0) were not observed after *in ovo* vaccination. However, the levels in the vaccinated group were significantly higher than those seen in the negative controls. Approximately one-fifth of the vaccinated birds had positive titers after 4 weeks of age. Therefore, at the time of challenge, overall seroconversion to ELISA levels above 6.0 may not be a good indicator of the protection actually afforded by the *in ovo* vaccination. Local immunity may play a role in the interaction between the protection induced and the experimentally-determined titers.

Prior to the analysis of Experiments 1-4 above, it had been postulated that the presence of maternal antibodies would adversely effect the effectiveness of vaccines. Example 4 nonetheless and surprisingly establishes that *in ovo* administration of a TRT vaccine at a titer of $10^{3.2}$ $TCID_{50}$ per egg was efficacious in reducing clinical TRT disease in chickens that are MA+. Example 4 strengthens and extends the conclusions reached in Example 3 above; namely, that SPF (MA-) chickens which were vaccinated *in ovo* likewise experienced a reduction of clinical disease.

Example 5: Study of TRT vaccine in combination with other poultry vaccines

The following abbreviations are utilized in this study:

AHS : Animal Health Service, Deventer, The Netherlands
BC : Biochek, Gouda, The Netherlands
CVL : Central Veterinary Laboratory, Weybridge, UK
EID50 :50% egg infective dose

ELISA	:	enzyme-linked immunosorbent assay
FAT	:	fluorescent antibody test
HI	:	haemagglutination inhibition
IB	:	infectious bronchitis
IBD	:	infectious bursal disease
i.m.	:	intramuscular(ly)
MD	:	Marek's disease
ND	:	Newcastle disease
P	:	probability
TCID50	:	50% tissue culture infective dose
TRT	:	turkey rhinotracheitis

The following vaccine materials were utilized for this study:

Poulvac® TRT, batches TR02100 and TR02200, containing 104.1 and 104.1 TCID50 TRT virus per dose, respectively.

Poulvac® Ovoline ND, batch BB010, containing 103.9 EID50 ND virus per vial (5000 doses per vial).

Bursamune IN OVO, batch 61640, containing 5000 doses per vial.

Poulvac® Marek HVT lyo, batch 350129, containing MD virus, strain FC126 (1000 doses per vial).

Poulvac® NDW, batches BL03200 and BL04302.

Poulvac® IB Primer, batch CX02301, containing 105.0 EID50 IB virus, serotypes M41 and D207 (D274 Clone), per dose (1000 doses per vial).

Poulvac® Bursa Plus, batch 62481, containing 103.2 EID50 IBD virus, strain V877, per vial (2000 doses per vial).

All vaccines, except for Bursamune IN OVO, were supplied by Fort Dodge Animal Health Benelux, Weesp, The Netherlands. Bursamune IN OVO was obtained from Fort Dodge Animal Health, Australia.

Vaccine batches were stored at 0 - 8 °C, protected from light, until the day of use and were used according to the manufacturer's specifications. After reconstitution, the vaccines were used within two hours. The following vaccine dilutions were prepared.

Vaccine dilution for vaccination in-ovo group 1 Poulvac® TRT, batch TR02100, Poulvac® Ovoline ND and Bursamune IN OVO were reconstituted and further diluted in Poulvac® Marek diluent, batch C8109. The final dilution contained one commercial dose of each vaccine in 0.05 ml.

Vaccine dilution for intramuscular (i.m.) vaccination groups 1 and 2 against MD
Poulvac® Marek HVT lyo was reconstituted and further diluted in Poulvac® Marek diluent, batch C8109. The dilution contained one commercial dose per 0.5 ml.

Vaccine dilution for coarse spray vaccination group 1 Poulvac® IB Primer was reconstituted and further diluted in 2.2 litres of demineralized water. The dilution contained one commercial dose per 0.5 ml.

Vaccine dilution for coarse spray vaccination group 2 Poulvac® NDW, batch BL04302, Poulvac® IB Primer, and Poulvac® TRT, batch TR02200, were

reconstituted and further diluted in 3 litres of demineralized water. The final dilution contained one commercial dose per 0.5 ml.

Vaccine dilution for vaccination group 2 against IBD in drinking water Three vials Poulvac® Bursa Plus were reconstituted and further diluted in tap water.

Vaccine dilution for vaccination groups 1 and 2 against ND by Atomist spray Poulvac® NDW, batch BL03200 was reconstituted and further diluted in demineralized water until the final dilution contained one commercial dose per 0.5 ml.

14895 commercial eggs of Cobb breed for broiler production, obtained from Pronk, Meppel, The Netherlands, were placed in one incubator located in MUK-5/6 at the test farm of Fort Dodge AHH, Muiden, The Netherlands. All eggs were candled after 17 days of incubation. Non-fertilised eggs or eggs with dead embryos were removed.

6001 Eggs assigned to group 1 were inoculated with 0.05 ml of a dilution containing 1 dose of Poulvac® TRT, Poulvac® Ovoline ND and Bursamune IN OVO per egg after 18 days incubation using an Embrex Inovoject egg injection machine according to the manufacturer's instructions. 6028 Eggs assigned to group 2 were left non-inoculated.

Inoculated (group 1) and non-inoculated (group 2) eggs were further incubated (without turning) until hatching in the incubators 1 and 4, respectively, located at the test farm of Fort Dodge AHH, Muiden, The Netherlands. After hatching, 4452 (inoculated, group 1) and 5248 (non-inoculated, group 2) chicks were included in the study, respectively. The chicks were group-housed on wood shavings in animal facilities located in the same shed at the test farm of Fort Dodge AHH, Muiden, The Netherlands. Chicks of group 1 were housed in MUL-L and chicks of group 2 in MUL-R. All

chicks were fed ad libitum with commercial broiler pellet and had free access to drinking water provided in bell drinkers.

The vaccination schedule is shown in Table 22. At one day of age, chicks of group 1 were vaccinated with Poulvac® IB Primer. One commercial dose was administered by coarse spray using a garden sprayer (Gardena) in 0.5 ml per chick. At one day of age, chicks of group 2 were vaccinated with Poulvac® TRT, Poulvac® NDW and Poulvac® IB Primer. One commercial dose of each vaccine was administered by coarse spray in one volume of 0.5 ml per chick, as in group 1. Before coarse spray vaccination, the chicks were placed in chicken boxes and were left therein until 3 hours post-vaccination.

All chicks were vaccinated with Poulvac® Marek HVT lyo, one commercial dose per chick, at one day of age. The vaccine was i.m. applied in the thigh, using a Pullet Injection Gun (Veterinary Supplies, Mijdrecht, The Netherlands) with a pre-set volume of 0.5 ml.

Chicks of group 2 were vaccinated with Poulvac® Bursa Plus, one commercial dose per chick in the drinking water, at 2 weeks of age. The vaccine was distributed in a quantity of water that was consumed within 2 hours. The chicks were deprived of drinking water during 2 hours before the vaccine was administered.

All chicks were vaccinated with Poulvac® NDW at 4 weeks of age. One commercial dose in 0.5 ml per chick was applied by atomiser.

Table 22. Vaccination schedule.

Group	Age at vaccination	vaccine	application method
1	eggs 18 days of incubation	Poulvac TRT) Poulvac Ovoline ND) Bursamune IN OVO)	injection machine
1	Chicks 1 day of age	Poulvac IB Primer) Poulvac Marek HVT lyo	coarse spray in chicken box i.m.
2	Chicks 1 day	Poulvac TRT)	coarse spray in

	of age	Poulvac NDW) Poulvac IB Primer) Poulvac Marek HVT lyo	chicken box i.m.
2	Chicks 2 weeks of age	Poulvac Bursa Plus	drinking water
1 and 2	Chicks 4 weeks of age	Poulvac NDW	atomiser

Blood samples were taken from 30 chicks of each group after decapitation at one day of age. Samples of 10 chicks of each group were used for serological tests at AHS. Samples of 10 other chicks of each group were used for serological tests at the Central Veterinary Laboratory, Weybridge, UK (CVL). The remaining 10 samples were used for serological tests at BC. Blood samples were collected from the wing vein up to a maximum number of 24 chicks per group at 2, 3, 4, 5 and 6 weeks of age.

Antibody titres to ND, IB M41 and IB D274 antigens were determined using HI tests. The detection limit of the HI tests corresponds with 2log HI titre = 1.0 for ND antigen and with 2log HI titre = 3.0 for IB antigens. Geometric mean HI titres were calculated. Antibody titres to TRT and IBD virus were measured using an ELISA method (IDEXX) according to the manufacturer's instructions and mean titres were calculated. These tests were done at AHS.

Antibody titrations to ND, IB, TRT and IBD virus using ELISA methods were done by BC. ELISA antibody titres of 1159 and 834 or above to ND and IB virus, respectively, are regarded positive. Antibody titres to ND (regarded positive at 396 or above) and IB virus were also determined using the IDEXX test kits. Mean titres were calculated using the BC99 software.

The results obtained at AHS and CVL were analyzed statistically.

The two groups were compared with respect to antibody titres by means of Student's 2-sided t test. ELISA antibody titres to IBD, TRT and MD were log transformed (log[x+1]) before analysis. The MD measurements ≥1600 and neg. were replaced by 1600 and 25 in the calculations, respectively. A probability of $P \leq 0.05$ was considered as a statistically significant difference.

Results: Mean antibody titres to ND, IB, IBD and TRT antigens, determined by AHS and BC, are shown in Tables 23 and 24.

Table 23. Mean antibody titres to ND, IB M41, IB D274, IBD and TRT, determined by AHS.

		mean 2log HI antibody titre			mean ELISA antibody titre	
age	Group	ND	IB M41	IB D274	IBD	TRT
one day	1	5.4 (n=10)	8.0 (n=10)	7.9 (n=10)	2377 (n=10)	4896 (n=10)
	2	5.8 (n=10)	8.1 (n=10)	7.9 (n=10)	3084 (n=10)	1628 (n=10)*
2 weeks, vaccination with Poulvac Bursa Plus (group 2)						
	1	2.3 (n=24)	6.2 (n=24)	6.5 (n=24)	94 (n=24)	509 (n=24)
	2	2.2 (n=24)	5.8 (n=24)	6.3 (n=24)	109 (n=24)	371 (n=24)*
3 weeks						
	1	2.5 (n=24)	5.4 (n=24)	5.6 (n=24)	51 (n=24)	203 (n=24)
	2	1.2 (n=23)*	5.4 (n=20)	5.4 (n=23)	45 (n=24)	90 (n=24)*
4 weeks, vaccination with Poulvac NDW (groups 1 and 2)						
	1	1.8 (n=24)	3.8 (n=23)	4.1 (n=24)	951 (n=24)	76 (n=23)
	2	1.3 (n=24)	3.7 (n=24)	4.0 (n=24)	1196 (n=24)	7 (n=24)*
5 weeks						
	1	3.6 (n=24)	4.6 (n=21)	4.9 (n=21)	1392 (n=24)	426 (n=24)
	2	1.8	4.0	4.2	1210	41 (n=24)*

		(n=24)*	(n=22)	(n=22)	(n=24)	
6 weeks						
	1	2.3	4.3	4.6	1907	215 (n=24)
	2	(n=24)	(n=24)	(n=24)	(n=24)	72 (n=24)
		3.3	3.7	3.8	1857	
		(n=24)*	(n=24)*	(n=24)*	(n=24)	

* statistically significantly different from group 1 ($P \leq 0.05$).

Table 24. Mean antibody titres to ND, IB, IBD and TRT, determined by BC.

		mean ELISA antibody titre			
Age	Group	ND	IB	IBD	TRT
one	1	7309 (n=10)	6316 (n=10)	4643 (n=10)	11144 (n=10)
day	2	7087(n=10)	6148 (n=10)	4601 (n=10)	2594 (n=10)
2 weeks, vaccination with Poulvac Bursa Plus (group 2)					
	1	1279(n=24)	497 (n=24)	399 (n=24)	804 (n=24)
	2	1088(n=24)	649 (n=24)	400 (n=24)	610 (n=24)
3 weeks					
	1	1934(n=24)	687 (n=24)	440 (n=24)	316 (n=24)
	2	763(n=24)	718 (n=24)	854 (n=24)	258 (n=24)
4 weeks, vaccination with Poulvac NDW (groups 1 and 2)					
	1	924(n=24)	1100 (n=24)	5448 (n=24)	298 (n=24)
	2	530(n=24)	680 (n=24)	6291 (n=24)	451 (n=24)
5 weeks					
	1	2918(n=24)	1041 (n=24)	6142 (n=24)	483 (n=24)
	2	1604(n=24)	752 (n=24)	5948 (n=24)	279 (n=24)
6 weeks					
	1	3074(n=24)	1435 (n=24)	6322 (n=24)	456 (n=24)
	2	2536(n=24)	2662 (n=24)	6801 (n=24)	681 (n=24)

Mean FAT titres to MD virus, representing the mean serum dilution demonstrating specific fluorescence, are shown in Table 25. Statistically significant higher FAT titres were observed in group 2 at 2, 3, and 4 weeks of age, showing a higher immunological response at younger age of the birds.

Table 25. Mean FAT titres to MD virus.

Age	group	no. samples	mean FAT titre to MD

			virus
one day	1	10	880
	2	10	940
2 weeks	1	24	66
	2	24	175*
3 weeks	1	24	172
	2	24	612*
4 weeks	1	24	369
	2	24	823*
5 weeks	1	24	950
	2	24	877
6 weeks	1	24	1129
	2	24	1267

* statistically significantly different from group 1 ($P \leq 0.05$).

Example 5 – Discussion

Chicks in both groups had MA against ND, IB, IBD, TRT and MD virus. A seroresponse to ND and IB was observed after vaccination at one day old. Development of titres to these antigens was within normal ranges in both groups. The second vaccination with Poulvac® NDW induced a seroresponse in both groups. Both groups developed antibody titres to IBD, TRT and MD virus.

In a number of cases, certain differences in antibody titres between groups was observed. Antibody titres to ND were higher in group 1 (chicks hatched from inoculated eggs) in all cases except at 6 weeks of age. Antibody titres to TRT were also higher in group 1 in all cases and these differences were statistically significant except for the difference at 6 weeks of age. The seroresponse to MD was somewhat slower in group 1 but reached the same

level as in group 2 at 5 weeks of age. No clear differences between groups in mean antibody titres to IB and IBD antigens were observed.

In most instances, the results of the titrations done at BC followed the same pattern as those obtained at AHS, with higher values for the ELISA titres to IBD and TRT. A seroresponse to IBD was shown by the BC test at 3 weeks of age, which is one week before it was shown in the IDEXX test. The BC ELISA test seems more sensitive to measure antibody titres to ND than the HI method. The BC ELISA test showed a more pronounced response to IB virus than the HI method from 3 weeks of age onwards.

In-ovo vaccination of commercial eggs for broiler production with one commercial dose of Poulvac® TRT, Poulvac® Ovoline ND and Bursamune IN OVO after 18 days of incubation followed by vaccination with Poulvac IB Primer and Poulvac® HVT at one day of age is compatible regarding efficacy with vaccination of the hatched chicks with commercial dosages of Poulvac TRT, Poulvac® NDW, Poulvac® IB Primer and Poulvac® Marek HVT lyo on the first day of life and with vaccination with Poulvac® Bursa Plus at 2 weeks of age.

The BC ELISA test kits seems favorable for measuring antibody titre levels to ND, IB, IBD and TRT viral antigens compared to HI tests to ND, IB M41 and IB D274, or IDEXX test kits to IBD or TRT.

Tables 26 through 35 provide additional antibody titre results.

Table 26. HI antibody titres to ND virus, determined by AHS.

age	Group	no. chicks with indicated 2log HI titre to ND virus									Insufficient Serum
		1	2	3	4	5	6	7	8	9	
one day											
	1				1	5	3	1			
	2				2	2	2	4			
2 weeks											
	1	6	12	3	1	1		1			
	2	2	16	6							
3 weeks											
	1	7	7	6	1	2		1			
	2	20	2	1							1
4 weeks											
	1	15	4	2	1	1	1				
	2	19	3	1	1						
5 weeks											
	1	4	3	6	4		7				
	2	12	7	4	1						
6 weeks											
	1	7	7	6	3	1					
	2	4	4	4	6	4	2				

Table 27. HI antibody titres to IB M41 antigen, determined by AHS.

age	Group	no. chicks with indicated 2log HI titre to IB M41 antigen									Insufficient Serum
		3	4	5	6	7	8	9	10	11	
one day											
	1				3		2	4	1		
	2					3	5	1		1	
2 weeks											
	1	1	3	3	5	8	4				
	2		1	7	11	5					
3 weeks											
	1	2	2	7	10	3					
	2	2	3	3	9	3					4

4 weeks											
	1	10	9	3	1						
	2	14	5	4		1					
5 weeks											
	1	7	3	6	3	1	1				3
	2	11	3	6	2						2
6 weeks											
	1	6	10	4	4						
	2	11	10	2	1						

Table 28. HI antibody titres to IB D274 antigen, determined by AHS.

age	group	no. chicks with indicated 2log HI titre to IB D274 antigen									Insufficient Serum
		3	4	5	6	7	8	9	10	11	
one day											
	1				1	4	2	2		1	
	2			1	2	2		3	1	1	
2 weeks											
	1		2	3	5	9	5				
	2			2	12	10					
3 weeks											
	1		4	5	12	2	1				
	2	1	4	6	8	4					
4 weeks											
	1	8	8	6	2						
	2	10	8	3	2	1					
5 weeks											
	1	7	2	3	6	1	2				3
	2	7	6	6	3						2
6 weeks											
	1	5	6	9	1	3					
	2	12	6	5	1						

Table 29. Individual ELISA antibody titres to IBD virus, determined by AHS.

ELISA antibody titre to IBD virus per group at various ages											
1 day		2 weeks		3 weeks		4 weeks		5 weeks		6 weeks	
group 1	group 2	group 1	group 2	group 1	group 2	group 1	group 2	group 1	group 2	group 1	group 2
1124	531	0	0	0	0	38	33	567	342	564	644
1547	1927	0	2	0	0	174	57	648	368	826	809
1799	2076	2	25	0	0	299	299	648	653	1038	1004
2388	2584	8	38	0	0	570	325	954	907	1132	1012
2421	2989	8	38	0	0	577	564	1005	986	1279	1462
2453	3355	8	51	0	0	637	660	1024	1119	1314	1497
2529	3466	13	57	0	0	721	690	1084	1167	1418	1524
2704	4096	13	57	0	0	729	744	1114	1339	1550	1533
3199	4550	19	64	0	0	751	886	1128	1339	1683	1674
3600	5262	25	64	0	0	805	919	1152	1378	1701	1728
		32	77	0	0	867	984	1183	1388	1719	1844
		44	77	3	0	883	1031	1243	1456	1871	1853
		44	111	15	3	929	1084	1247	1460	1888	1897
		51	118	19	3	953	1141	1257	1577	1969	1987
		57	118	57	8	968	1185	1538	1577	1987	1906
		105	125	65	15	976	1261	1716	1972	2078	1996
		118	125	80	15	984	1269	1762	1987	2195	2014
		147	161	104	28	1000	1430	1767	448	2250	2123
		161	168	137	28	1078	1690	1873	613	2387	2214
		175	182	137	28	1567	1808	1875	1179	2432	2250
		234	197	137	113	1681	2009	1903	1217	2885	2314
		271	212	146	182	1772	2326	1993	1281	2968	2497
		278	234	154	214	1862	2362	2062	1308	3033	3005
		446	316	162	434	2011	3951	2654	1981	3616	3787

Table 30. Individual ELISA antibody titres to TRT virus, determined by AHS.

ELISA antibody titre to TRT virus per group at various ages											
1 day		2 weeks		3 weeks		4 weeks		5 weeks		6 weeks	
group 1	group 2	group 1	group 2	group 1	group 2	group 1	group 2	group 1	group 2	group 1	group 2
6167	1627	465	0	0	1134	85	46	316	0	0	0
5889	2936	0	0	0	0	85	0	0	0	0	0
6096	4819	371	602	0	0	0	0	0	0	19	0
3542	1457	65	0	0	0	0	0	0	0	709	0
1036	1853	0	1686	0	0	153	0	342	0	0	0
3305	681	19	0	0	0	153	0	0	0	380	337
1784	295	371	0	0	0	0	0	342	0	204	161
7540	1332	2504	0	65	414	0	0	0	931	342	69
3899	840	227	740	241	0	0	0	781	0	123	0
9698	442	711	1612	346	0	383	0	19	0	0	0
		346	0	213	0	55	0	275	0	881	0
		565	0	0	376	0	0	709	0	0	69
		0	0	0	0	0	0	289	0	0	500
		1876	0	465	0	0	0	380	0	0	0
		0	0	0	0	0	126	106	0	0	0
		103	368	0	0	0	0	45	0	262	0
		0	1669	442	0	0	0	0	0	1669	0
		840	289	442	0	0	0	2620	0	0	0
		120	0	499	0	0	0	1289	0	0	0
		0	881	741	0	0	0	761	46	219	0
		499	1063	1008	89	169	0	852	0	204	365
		1161	0	227	0	282	0	781	0	0	89
		1547	0	103	144	454	0	204	0	0	144
		430	0	85	0	0	0	123	0	140	0

Table 31. Individual FAT titres to MD virus, determined at CVL.

FAT titres to MD virus per group at various ages											
1 day of age		2 weeks of age		3 weeks of age		4 weeks of age		5 weeks of age		6 weeks of age	
group 1	group 2	group 1	group 2	group 1	group 2	group 1	group 2	group 1	group 2	group 1	group 2
800	≥1600	100	200	100	400	100	≥1600	800	200	800	800
400	800	50	400	neg.	400	200	800	800	50	≥1600	800
≥1600	800	50	200	100	200	100	200	400	≥1600	800	800
800	800	neg.	400	400	≥1600	200	≥1600	800	≥1600	400	800
400	200	100	400	50	400	100	400	200	400	800	800
800	≥1600	100	200	50	800	800	800	≥1600	≥1600	800	≥1600
400	400	100	100	50	800	200	≥1600	800	400	800	≥1600
≥1600	≥1600	100	100	50	≥1600	50	800	400	800	≥1600	≥1600
≥1600	800	50	50	400	800	800	800	400	800	≥1600	800
400	800	50	200	400	400	100	800	≥1600	800	800	800
		50	100	100	800	400	100	200	≥1600	≥1600	800
		100	200	200	200	50	400	≥1600	400	≥1600	≥1600
		50	100	400	100	100	≥1600	800	800	≥1600	800
		50	200	200	≥1600	800	800	≥1600	800	≥1600	800
		50	200	50	400	100	≥1600	≥1600	800	≥1600	≥1600
		100	200	400	200	100	200	400	≥1600	400	≥1600
		100	200	200	200	200	400	≥1600	800	≥1600	≥1600
		50	200	100	200	800	≥1600	800	≥1600	400	≥1600
		50	50	200	≥1600	400	400	400	400	≥1600	≥1600
		100	100	100	400	≥1600	800	800	800	100	≥1600
		50	100	200	200	400	50	400	400	≥1600	≥1600
		neg.	100	200	200	400	400	≥1600	≥1600	200	≥1600
		50	100	50	400	50	≥1600	≥1600	800	≥1600	≥1600
		neg.	100	100	800	800	400	≥1600	400	≥1600	≥1600

Table 32. Individual ELISA antibody titres to ND virus, determined by BC.

ELISA antibody titre to ND virus per group at various ages											
1 day		2 weeks		3 weeks		4 weeks		5 weeks		6 weeks	
group 1	group 2	group 1	group 2	group 1	group 2	group 1	group 2	group 1	group 2	group 1	group 2
6742	6546	901	1126	2507	258	1338	215	705	1563	3275	818
9414	8225	454	550	490	175	500	175	1954	351	543	4924
7434	6129	1689	576	245	175	179	2169	4934	6719	9252	377
10709	10884	1123	384	6093	199	262	235	1172	596	434	1623
5639	7348	1808	977	2278	2202	818	149	5709	1606	1427	1225
1874	5096	864	1185	73	182	162	235	1964	1682	2010	9378
9248	9033	1152	242	1957	1252	520	894	3457	2477	4477	1401
6510	4179	1199	1652	5828	325	401	702	2119	285	4076	818
7530	4997	735	801	215	225	5030	1960	3695	394	3623	3265
7990	8434	460	2477	209	149	2179	242	1864	801	3858	2374
		1116	1010	5768	199	30	149	3176	242	2536	6990
		1427	1785	126	4503	1533	182	1334	3884	586	791
		1132	1652	2745	967	1063	1543	9305	1046	1136	884
		1921	669	245	626	901	818	6090	2308	1079	149
		752	775	215	225	871	50	222	4060	3192	139
		1457	1669	639	566	745	318	3146	623	3291	3146
		656	1099	1927	325	172	268	4222	1096	5205	3053
		1073	1709	113	199	1182	182	1179	977	4149	944
		3729	626	7477	242	1301	50	1079	437	8639	5305
		656	583	2070	132	66	450	616	1334	5679	937
		2715	917	447	242	179	460	6364	1470	2010	5424
		983	993	3997	318	371	182	2073	877	1589	2374
		2344	1093	430	325	96	126	3566	123	871	4467
		358	1553	321	4295	2285	977	86	3536	851	63

Table 33. Individual ELISA antibody titres to IB virus, determined by BC.

ELISA antibody titre to IB virus per group at various ages											
1 day		2 weeks		3 weeks		4 weeks		5 weeks		6 weeks	
group 1	group 2	group 1	group 2	group 1	group 2	group 1	group 2	group 1	group 2	group 1	group 2
3606	4019	609	700	671	504	496	354	1401	113	484	2230
8858	7858	71	179	496	629	233	329	4331	1071	375	338
8041	10238	671	629	671	434	596	434	1342	1784	442	538
7254	7199	171	296	321	308	233	225	513	263	171	3152
5386	6366	684	1105	221	1080	1296	179	825	338	4715	834
8458	3231	321	388	271	271	2639	746	267	438	484	634
4206	8296	896	504	521	700	196	1209	1863	1284	375	8075
3177	2364	534	1059	784	354	1751	1034	592	475	7212	1547
8267	6620	384	342	846	296	3039	1151	1384	163	117	3539
5911	5290	158	688	721	1255	1034	571	429	488	1276	6257
		634	363	258	850	271	1601	742	1109	909	1372
		984	988	959	1000	971	271	1984	475	5603	7141
		521	667	521	2168	2526	988	1251	2943	1042	1684
		496	817	258	467	884	342	742	1346	934	288
		434	642	371	780	634	1255	975	1672	484	1109
		521	467	333	688	884	446	1793	1447	3460	1409
		384	780	1547	504	2289	780	346	200	1888	4786
		796	1392	1676	513	308	192	688	659	1984	1868
		183	1255	233	388	1184	1000	567	250	308	859
		1109	563	1184	296	671	805	333	350	1342	1034
		271	434	846	780	1713	133	525	450	158	5920
		296	342	221	513	584	513	1492	375	196	3139
		371	513	1009	1776	596	805	363	188	225	5619
		434	467	1559	667	1372	967	238	175	267	513

Table 34. Individual ELISA antibody titres to IBD virus, determined by BC.

ELISA antibody titre to IBD virus per group at various ages											
1 day		2 weeks		3 weeks		4 weeks		5 weeks		6 weeks	
group 1	group 2	group 1	group 2	group 1	group 2	group 1	group 2	group 1	group 2	group 1	group 2
4992	3837	352	674	129	166	8581	184	5384	7522	8305	6358
6396	3265	215	436	33	115	9353	7700	4798	6419	4698	7756
5302	4215	129	352	76	2558	3938	9419	5133	12583	7539	7472
8986	7267	730	45	26	29	1071	11617	4971	4881	7152	13687
2112	5817	926	528	50	100	59	8172	6510	3848	4612	5131
1829	5150	582	511	8044	174	121	4568	7488	7006	5047	5850
7548	3613	687	192	76	2188	3574	4800	11164	5825	5373	7140
1970	3624	644	769	186	158	2786	9591	4971	4383	7410	6833
3898	2243	323	174	352	201	5652	4340	5762	8615	3090	5946
3400	6978	76	1088	149	115	7776	6419	8829	333	7023	8192
		323	133	26	6777	7469	8866	3925	6225	8818	9620
		121	606	79	3455	9341	6336	5095	4822	7435	6261
		129	380	26	100	938	6992	6947	9934	3450	5789
		323	473	79	29	10310	5289	5825	5191	5398	2334
		50	462	94	45	551	5474	5436	6688	8424	3592
		404	316	102	108	4595	7435	4389	7533	7295	4868
		243	380	264	93	9600	5828	5248	6627	6156	5523
		344	325	18	38	8365	4090	5674	7006	8073	6128
		753	52	50	29	9069	6491	9992	5861	5185	10466
		215	209	205	68	5368	6444	6446	6103	6372	6297
		571	235	94	115	2714	11605	5839	3893	5811	8840
		139	425	79	2	5562	2047	5523	5047	6588	6945
		861	262	112	3703	5781	2755	5471	2953	6065	7104
		425	568	205	133	8177	4523	6599	3466	6421	5095

Table 35. Individual ELISA antibody titres to TRT virus, determined by BC.

ELISA antibody titre to TRT virus per group at various ages											
1 day		2 weeks		3 weeks		4 weeks		5 weeks		6 weeks	
group 1	group 2	group 1	group 2	group 1	group 2	group 1	group 2	group 1	group 2	group 1	group 2
14811	1295	983	179	560	371	821	636	258	258	33	507
18017	3530	99	66	195	46	225	248	344	354	215	583
17444	6109	368	1334	99	401	238	513	460	497	517	1099
6566	5815	298	103	427	103	268	136	248	172	536	2023
19653	3338	99	2990	99	56	195	159	828	66	149	646
2626	1854	99	169	722	103	17	103	397	76	53	583
6285	288	146	219	56	136	99	401	560	291	129	411
1325	1162	4086	248	338	656	166	2119	119	76	20	497
7152	1467	447	626	540	169	318	626	86	225	387	119
17563	1083	470	1844	288	46	530	921	225	732	440	1281
		480	209	166	483	407	533	685	593	1729	884
		417	788	56	798	983	705	1609	205	526	1099
		99	56	99	474	146	298	685	507	387	689
		4460	103	629	103	99	7	291	368	656	152
		99	103	99	7	579	228	106	195	106	646
		156	877	298	331	510	411	20	109	20	517
		447	228	540	106	407	169	387	109	1556	430
		1132	513	268	66	175	563	987	464	924	550
		348	381	136	533	288	411	1278	421	235	656
		96	1083	679	103	298	381	656	368	772	1023
		530	868	649	126	17	666	546	205	934	689
		1146	17	99	103	99	103	526	195	33	626
		2083	321	156	778	99	228	33	109	546	517
		712	1305	397	96	166	258	258	109	53	109

Although the present invention has been described above in considerable detail, applicants desire the full extent of patent protection possible as defined and determined by the claims herein set forth, with reference to the above teachings but not limited to any particularly disclosed example, and in all events, consistent with the widest possible scope of the claims consistent with the spirit and scope of this application.